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EFEITOS COMBINADOS DE QUÍMICOS E RADIAÇÃO ULTRAVIOLETA EM *Daphnia magna*

COMBINED EFFECTS OF CHEMICALS AND ULTRAVIOLET RADIATION ON *Daphnia magna*

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Aos meus tios

o júri

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resumo

O ambiente natural está frequentemente exposto a vários tipos de estresses, que podem ser de carácter químico, físico ou biológico, originados da actividade humana e dos processos de alteração climática. Os pesticidas são geralmente usados em práticas agrícolas para controlar doenças em vegetais e o aparecimento de pragas, e podem ser levados do solo para os sistemas aquáticos adjacentes aos locais de aplicação, onde representam um factor de stress para os organismos não-alvo. Além das exposições a químicos, o ambiente está sofrendo as consequências dos processos de alterações climáticas. Uma destas consequências é o aumento da radiação ultravioleta que chega à superfície terrestre devido à diminuição da concentração de ozono na estratosfera. O presente trabalho teve como objectivo principal elucidar alguns padrões e comportamentos biológicos relativamente a mudanças no ambiente. Para isto, com o intuito de prever as interacções entre stressores naturais e químicos, a radiação ultravioleta (RUV) e o fungicida carbendazim foram escolhidos como fontes de stressores natural e químico, respectivamente, e foram aplicados em combinação, como um exemplo das possíveis condições adversas que podem ser encontradas no ambiente. Os efeitos isolados da radiação ultravioleta em Daphnia magna foram avaliados através da utilização de uma lâmpada artificial de RUV, à qual os organismos foram expostos por um período máximo de 5 horas. Os experimentos de combinação entre RUV e carbendazim foram conduzidos com exposição constante ao químico, e uma única dose de radiação ultravioleta. Os parâmetros analisados foram sobrevivência, actividade alimentar, reservas energéticas e produção de juvenis de Daphnia magna. Para prever os efeitos das combinações, um dos modelos utilizados na análise de misturas de químicos e combinação de químicos com stressores naturais foi o utilizado. O modelo da Acção independente (AI) assume que ambos os componentes da combinação têm diferentes modos de acção, e actuam de forma independente sobre o organismo. Os efeitos são avaliados de acordo com as probabilidades de não-resposta do organismo a ambos os componentes da combinação. Há ainda outras formas de interacção entre os componentes da combinação que podem produzir um efeito mais severo (sinergismo) ou menos severo (antagonismo); os efeitos podem ser também dependentes do nível da dose aplicada ou do rácio entre os dois componentes da combinação Os resultados da exposição de Daphnia magna à radiação ultravioleta somente demonstraram um decréscimo na sobrevivência, na actividade alimentar e na produção de juvenis, com valores de dose-efeito muito próximos para todos os parâmetros, o que pode ser explicado pela diferença da sensibilidade deste organismo à radiação, de acordo com a idade em que são expostos. Os resultados das combinações entre carbendazim e RUV para o parâmetro sobrevivência foram bem ajustados ao modelo da acção independente, e não demonstraram nenhum desvio. Para a reprodução e a actividade alimentar, houve um desvio dependente do rácio entre os componentes, que demonstrou maior toxicidade para o carbendazim quando a radiação ultravioleta era o item dominante na combinação. Este estudo mostra a importância da avaliação de combinações entre químicos e stressores naturais. Neste caso, espera-se que o aumento na radiação aumente a sensibilidade dos organismos, como a Daphnia magna quando expostos a stressores químicos, como o fungicida carbendazim.

keywords ultraviolet radiation, carbendazim, *Daphnia magna*.

abstract

The natural environment and wildlife are often exposed to several chemicals, physical and biological stressors originated from human activities and climate changes. Pesticides are often used to control plant disease and pest in agricultural practices, and can runoff from the soil to adjacent aquatic systems, where it represents a stress factor for non-target organisms. In addition to chemical exposures, the natural environment is suffering from climate change processes. One of the consequences of that is the increasing amount of ultraviolet radiation reaching the earth's surface due to depletion on stratospheric ozone. The present work aimed to elucidate some biological behaviors and patterns regarding changes in the environment. For that, to predict interactions between natural stressors and toxicants to Daphnia magna, the ultraviolet radiation (UVR) and the pesticide carbendazim were chosen as the source of natural and chemical stressors, respectively and were employed in combination with each other as an example of possible stress conditions that can be found worldwide in the environment. Single effects of ultraviolet radiation on Daphnia magna were assessed using an artificial UV source, by exposing the organisms to UV and visible light simultaneously, to a maximum period of 5 hours. Combined experiments of carbendazim and ultraviolet radiation were conducted with a constant chemical exposure and a single UVR dose. The parameters analyzed were survival, feeding activity, energy budget and offspring production of Daphnia magna. To predict effects of combined exposures, one of the reference models used for analysis of mixture toxicity and combination of chemical and natural stressor was applied. The Independent Action (IA) model assumes that both components of the combination have different modes of action, and act independently from each other; the effects of the combinations are based on the probabilities of non-response of the organism to both stressors. There are some deviations from the independent action model which can cause a more severe effect (synergism), or a less severe effect (antagonism); they might be also dose-level dependent or dose-ratio dependent. Results from single exposure of Daphnia magna to ultraviolet radiation showed a decrease in survival, feeding activity and offspring production, with similar dose-effect values, due to differences in the sensibility of the organism to UVR according to their age. Combined exposures of carbendazim and UVR for survival endpoint fitted to the IA model, showing no deviation patterns, while the response of reproduction and feeding activity were dose-ratio dependent, indicating a higher toxicity of carbendazim when ultraviolet radiation was the dominant item in the combination. This study shows the importance of evaluate the combined effects of chemicals and natural stressors. In this case, UVR increase is expected to enhance the sensitivity of organisms as D. magna when exposed to chemical stressors like the fungicide carbendazim.

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Chapter I

General Introduction and Objectives

I. General introduction

The natural environment and wildlife are often exposed to several chemicals, physical and biological stressors originated from human activities and climate changes. One of the anthropogenic activities that have a high impact on ecosystems is agricultural practices, because of the large use of pesticides in order to control plant diseases and pest. These chemicals are designed according to their target organism, and exist in a variety of types and classes. Pesticides, once applied in the soil, can runoff to adjacent aquatic systems, potentially representing a risk for non-target organisms (e.g. algae, aquatic invertebrates and fish). The fate of pesticides in the environment is linked with some natural properties, like precipitation levels (high rate of precipitation can lead to more runoff of chemicals from the soil), temperature, dissolved oxygen levels and ultraviolet radiation income, which are closely related to chemical degradation, bioavailability and volatility. (Noyes et al. 2009). Climate change phenomenon is leading to alterations of precipitation levels, increases of temperature and decrease of stratospheric ozone concentration, which acts as a filter to the shortest ultraviolet rays from the sunlight. As a consequence, the environment and wildlife is now experiencing a combination of several natural stressors, in addition to the already mentioned chemical exposure.

Environment risk assessment (ERA) usually evaluates effects of single stress exposures, and environment management regulations have been established based on that. Nowadays, there is an increase and urgent need to evaluate the combined exposures of stressors to obtain a more realistic prediction of environment risk.

In this work, a situation where the combination between a pesticide and increasing levels of ultraviolet radiation input to the water system was carried out in laboratory conditions, with the purpose to analyse the biological response of organisms to both stress sources.

Ultraviolet Radiation

Ultraviolet Radiation (UVR) is composed by wavelengths below 400nm, between X-rays and visible radiation, and divided into UV-A, or long wave (from 320nm to 400nm), UV-B, or medium wave (from 320 nm to 280) and UV-C, short wave or germicidal (from 280nm to 100nm). From these wavelengths, the UV-A and UV-B ranges can reach the earth's surface, and the UV-C is completely filtered by ozone layer present in the atmosphere (Madronich et al. 1998). The germicidal denomination for UV-C wavelengths is usually employed due to its action on microorganisms; this wave-range has the shortest and thus more energetic rays capable to damage and/or destroy microorganism's cells and DNA, and stop their reproductive ability.

The amount of UVR that reaches the earth's surface is determined by a series of factors interacting with the radiation as it passes through the atmosphere. These include the state of the atmosphere, position on the earth (latitude and altitude) and season (relative position of the sun to location on Earth) (Blumthaler and Webb 2003). Besides that, when reaching aquatic and/or terrestrial ecosystems, the UVR light is also modified by natural factors. For instance, in aquatic environments there are several natural features to which the solar radiation input can interact with. Dissolved organic carbon (DOC) and various humic substances present in ocean and lakes contribute tremendously for the penetration of UV radiation in the water column by absorbing the shortest UV wavelengths and blocking the amount of harmful radiation coming to the organisms. DOC is degraded by a slowly process in water, but it can be broken down by solar UVR and became more available for the bacterioplankton's metabolism (Moran et al. 2000) which leads to an increase in lake's UVR transparency (Madronich, McKenzie et al. 1998).

Dissolved organic matter (DOM) is the designation for the carbon-containing compounds derived from the decomposition of dead organisms that are present in aquatic systems. The chromophoric DOM (cDOM) absorbs the radiation that incomes aquatic ecosystems in the UV and blue ranges of the solar spectrum. The presence of cDOM gives to natural waters a yellow-brown colour. The fate of UV-R in lake system as been demonstrated to be dependent on the amount of DOM present in natural waters due to the attenuation of light penetration, or to effects on phytoplankton photosynthesis, which ultimately change the

amount of DOM (Häder et al. 2007). Williamson et al. (2009) has argued that the quantity and quality of DOC in inland waters is much more likely to control UV transparency than the ozone itself, meaning that although environmental policies for healing the ozone layer are showing beneficial results, other climate change factors can interfere with harmfulness of UV radiation income, mainly changes in DOC concentration that leads to more UV transparent waters.

UV-R can cause indirect effects on freshwater nutrient cycles by breaking down the DOC into dissolved inorganic carbon (DIC). When exposing the macrophyte *Vallisneria gigantean* to UV-B levels, Anusha (2008) observed that at the end of ten weeks, the amounts of DOC and DIC had a significant decrease and increase, respectively. The transformation of DOC into DIC by UV-R increases the microbial population due to the availability of inorganic carbon substrates and increasing bacterial population are likely to decompose organic carbon under environmental sunlight (McCallister et al. 2005). In addition to decrease of stratospheric ozone concentration that leads to increase of UVR input, climate change events can also cause severe situations of drought or flood, depending on precipitation levels alterations (Whetton et al. 1993; Le Houérou 1996) Drought reduces the export of terrestrially-derived CDOM to aquatic ecosystems and may also influence water export from wetlands that are important determinant of CDOM inputs to other downstream aquatic ecosystems (Williamson et al. 2003). Flood can increase the rate at which anthropogenic-introduced compounds in agricultural areas are leachate or run off to adjacent water bodies.

UV effects on aquatic organisms and counteracting strategies

Phytoplankton and macrophytes

Phytoplankton represents the major primary producers in oceans, being the basis of food webs. The sensibility of phytoplankton to ultraviolet radiation is reported to ecosystems from tropical to polar regions (Helbling et al. 1994). Phytoplankton are potentially subject to harmful UV-B radiation which can cause DNA damage, inhibited photosynthesis and growth, and finally, cell death (Klisch et al. 2001). UVR is likely to alter photosynthesis through photo-inhibition, at relatively high doses; some studies have

shown that UV-A causes more photosynthetic inhibition rather than UV-B because their natural levels are higher. UVR potentially impair the performance of the three main photosynthesis component processes: photophosphorylation reactions, on the thylakoid membrane, the CO_2 fixation reactions of the Calvin cycle and stomatal control of CO_2 supply (Allen et al. 1998).

This inhibition is highly variable, depending on the irradiance/dose received by the cells, the sensibility of the cell and other environmental factors that can mask the observed effects (Villafaiie et al, 2003). The mechanism in which UVR impairs photosynthesis is linked with the bleaching of photosynthetic pigments. Freshwater phytoplankton are likely to be more inhibited by solar UV than marine phytoplankton (Häder, Kumar et al. 2007). Cyanobacteria, as well as phytoplankton and macroalgae can synthesize ultraviolet absorbing/screening compounds that can absorb the radiation before it can reach intracellular targets. One of the most common compounds is mycosporine-like amino acids (MAAs), which has a maximum absorption range from 310 to 360nm. (Klisch, Sinha et al. 2001).

Artificial UV-B was shown to induce the activity of nitrate reductase in cyanobacteria; for these organisms, the primary photosynthetic reactions and CO_2 uptake is affected by UV-B (Häder, Kumar et al. 2007).

Macrophyte species play an important role on nutrient cycling in freshwater systems. One of the consequences related to the inhibition of photosynthesis caused by UVR is the decrease of dissolved oxygen concentrations in shallow freshwater type systems (Anusha and Asaeda 2008).

Zooplankton

Zooplankton species have a major role in aquatic food chains, and alterations in any level (individual, population and community) can lead to changes in aquatic systems functionality. For instance, the decrease in filter-feeding species can generate accumulation algae cells in lakes during the late spring and early summer seasons; moreover, changes on zooplankton community represents depletion on food source for planktivores fishes (Confer et al. 1978). For that reason, zooplankton are widely used in ecotoxicology testing with the objective of predict environmental risk. As climate changes introduce alterations

in environmental components and compartments, zooplankton species as been used for the assessment of natural stressors effects in freshwater environments, mainly the role of increasing ultraviolet penetration through water column (Rhode et al. 2001; Alonso et al. 2004; Marinone et al. 2006). In earlier researches, the vertical migration of zooplankton was believed to happen only due to visual predation avoidance (Zaret and Suffern 1976). Nowadays, the UVR exposure is assumed to be an important factor that guides the vertical migration of zooplankton into deeper waters (Boeing et al. 2004; Fischer et al. 2006b). In daphnia species case, UV-tolerance is linked with pigmentation; melanin pigments as well as carotenoids increase their tolerance to UVR (Herbert and Emery 1990). Rhode and co-workers (2001) observed a deeper distribution of *Daphnia* that presented low pigmentation, under both laboratory and natural sunlight exposures. In order to minimize and balance metabolic costs of deeper cold waters, a less pronounced downward migration is predict for zooplankton with higher UV-R tolerance (Rhode, Pawlowski et al. 2001). Moreover, for the high-pigmented *Daphnia* living in low DOC lakes, there is a conflicting factor of visual predation by fish (Confer, Howick et al. 1978).

The main target of UVR in organisms is the DNA molecule (Teoule 1987; Huot et al. 2000). UV-B can cause dimerization of DNA bases, leading to the formation of cyclobutane pyrimidine dimmers (CPDs) and 6-4 pyrimidine photoproducts (PPs). These photoproducts block DNA transcription and replication as only a single distortion of DNA may be sufficient to stop DNA replication (Buma et al., 2003). The molecular repair of these damages is strictly dependent on UV-A and visible light. In general, there are two types of DNA repair described for organisms: nucleotide-excision repair (NER) and photoenzymatic repair (PER). NER is a complex and multi-enzimatic process, which depends on energy provided by ATP. Therefore, it is a metabolic cost process to the cell (de Laat et al. 1999). PER is performed by one single enzyme, the photolyase and requires energy from visible light and UV-A range (Carell et al. 2001). PER is specific for repair damage caused by ultraviolet light, and it is found in many diverse species, such as zooplankton, fishes and bacterioplankton (Huot, Jeffrey et al. 2000; Gonçalves et al. 2002; Dong et al. 2008). Considering the enzymatic nature of DNA photo-repair, temperature has a crucial role in this process as in all other enzymatic mechanisms. MacFadyen (2004) observed that ectotherms that depend on temperature-dependent enzyme processes might

be less able to repair DNA damages at low temperatures, and argued that low temperatures are likely to favor photoprotection rather than photorepair.

Zooplankton also shows impairments in reproduction caused by UVR exposure as a consequence of metabolic alterations. Karanas et al (1981) observed that when the copepod *Acartia clausii* survived to UV radiation exposure, its ability to reproduce was impaired. Another study showed differences in survival and reproductive output of *Daphnia magna* aging from 1 to 4 days during the post-exposure period to UVR (Huebner et al. 2006). Lacuna and Uye (2000) also described a marked reduction on gut content and egg production of *Sinocalanus tenellus* females that were exposed to high sub-lethal doses of UV-B. Changes in respiration rates of *Daphnia catawba* pre exposed to ultraviolet radiation were related to metabolic costs of DNA repair as described by Fischer et al (2006a) Another consequences aroused from zooplankton exposure to UVR is the formation of reactive oxygen species (ROS) causing oxidative stress. Vega and Pizarro (2000) observed an increase on catalase activity in *Daphnia longispina* exposed to UV-B radiation, probably as a strategy to avoid oxidative stress. Oxy-radicals might lead to tissue damages through lipid peroxidation, eventually causing impairments of vital cellular functions and alterations in physicochemical properties of cell membranes. (Barata et al. 2005).

Daphnia magna Straus, 1820 as a model organism for ecotoxicology tests

The genus Daphnia is one of the most widely known groups of freshwater invertebrates. Many member species are used as model-organisms in ecotoxicological tests. The genus is most diverse and abundant in temperate regions, but it has representation through all climate zones and continents, and is one of the dominant members of the world's freshwater zooplankton. Daphnia reproduces largely by cyclic parthenogenesis; females lay eggs into the brood pouch that do not require fertilization, and which produces only females; after many cycles of this asexual process, and usually in response to adverse environmental conditions (e.g., temperature, population density, pH, etc), the parthenogenetic females produce males or mixed brood of male and female. Some evidences suggests that the induction of sexual females can be due to changes in photoperiod, food levels or crowding while males can be induced by photoperiod or by a chemical sign emitted when there is high density of females. (Ferrari and Hebert 1982).

Sexual females produce haploid eggs which are fertilised by males and develop diapausing embryos, encased in a protective structure called ephippium. These resting eggs can be hatch when the conditions became favorable again, and during the dorment time they might have been dispersed to other location. Sexual reproduction provides a generation of novel genotypes through recombination. Individuals that hatch from epphippia not only have the potential to survive in unfavorable conditions, but also the sexual process increases the probability that some of the new genotypes produced are better adapted to novel environments conditions in which the eggs can hatch.

After released into the brood pouch, parthenogenic eggs and the ones destined to become males, develop juveniles. Once neonates are released to the external environment, they go through five events of moulting to become mature; after which they continue to growth. Moulting process also happens after each occasion of brood release. Daphnids feed on both live and dead suspended matter, including protozoa and bacteria, but mainly phytoplankton. Organisms inhabiting shallow ponds can feed on material settled to the bottom by creating water currents with the beating of their thoracic limbs to suspend the matter, and afterwards by filtering it.

Members of the genus Daphnia posses a large distribution and can be often found in freshwater and continental saline lakes, but is not found in the marine environment. Temperate and higher latitudes are likely to present the most diversity of *Daphnia* species. Both abundance and number of species are decreased in tropic regions, and for this areas, higher latitudes and thus lower temperatures are the most often places of records of Daphnia species. Species living on clear waters at high latitudes have developed intense pigmentation as a protection from high UV radiation levels.

The large applicability of *Daphnia magna* in laboratory assays is due to several reasons. Among them are the small size and short life-cycle of the species, which allows to obtain new organisms in a relatively short time period, and do not demand large spaces for cultures. Another feature is the significance of this species to the ecology of freshwater trophic chains and the overall environment.

Chemical compounds

Carbendazim

Carbendazim (methyl-2-benzimidazole carbamate) is a fungicide that belong to the benzimidazole carbamate class with a wide applicability in agricultural activity against fungal diseases.

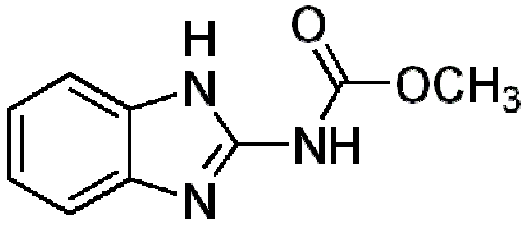

Figure 1. Methyl *1H*-benzimidazol-2-ylcarbamate structure.

Carbendazim is a metabolite of thiophanate-methyl, which breaks down rapidly in the environment, generating carbendazim (A.P.V.M.A 2007). The fungicide is used to control a broad range of diseases on arable crops (cereals, oilseed rape), fruits, vegetables and ornamentals. It is also used in post-harvest food storage, and as a seed pre-planting treatment. Carbendazim acts by interrupting the development of fungal germ tubes, the formation of appressoria and the growth of mycelia (A.P.V.M.A 2007). Effects on non-target individuals has been related to impairments on cell division and to the inhibition of the enzyme acetylcholinesterase (Cuppen et al. 2000). Mitosis in plants and mammalian cells is affected by carbendazim by the impairment of formation and functioning of microtubules (Davidse 1977). Ferreira et al. (2008) observed that carbendazim affects feeding activity of *Daphnia magna* at concentrations above 70µg/L, with 50% of reduction near 100µg/L. In the same study, a LC_{50} value of this fungicide to *Daphnia magna* was established at 156µg/L. The photochemical behaviour of carbendazim in aqueous solution was investigated and a relationship with environmental characteristics was observed; the photodegradation rate is accelerated in alkaline solutions, and under UV radiation levels, while under natural sunlight, carbendazim showed to be a stable compound (Boudina et al. 2003). Carbendazim affects the structure of aquatic ecosystems indirectly, by promoting increase in abundance of phytoplanktonic algae, through impairments on grazing pressure by zooplankton in a microcosm experiment, as observed by Van den Brink et al (2000).

Predictions of joint effects of natural stressor and chemical compounds

In natural systems, wildlife species are often exposed to mixtures of chemical compounds and combinations between chemical and natural stressors. Climate change leads to alterations of environmental natural conditions, such as temperature, precipitation, increase of ultraviolet radiation input, polar ice melting, water acidification, changes on carbon cycling, etc. (Justic et al. 1996; Koinig et al. 1998; Parmesan and Yohe 2003). The effects of single natural stressors have being evaluated for aquatic and terrestrial species; for instance, effects of increasing amount of ultraviolet radiation, temperature and decrease of dissolved oxygen have been reported for some zooplankton species (Häder, Kumar et al. 2007; Ferreira, Loureiro et al. 2008), as well as the effects of several single chemical exposures that occur in aquatic systems. Indeed, the environmental risk assessments is now taking into consideration that possible mixtures of chemicals and combinations of chemicals and natural stressor are crucial when evaluating ecological and human risk.

For the prediction of joint toxicity, an approach based in two foundation concepts has been used for chemical mixtures but also for the combined effects of chemicals and natural stressor (Kienle et al, 2008). First, if chemicals stressors are believed to have the same mode of action, their combined toxicity will be described by the concentration addition model (CA); on the other hand, if they present dissimilar modes of action, the independent action model (IA) is used to predict the joint toxicity. Concentration addition was first formulated by pharmacologists in 1926 (Loewe and Muischnek, 1926) and is based on the idea that all chemicals in the mixture will act by the same mode of action, i.e. on the same target of the organism. The CA model is mathematically expressed by the formula:

$$\sum_{i=1}^{n} Ci / ECi = 1$$

Where C_i is the concentration of the chemical *i* in the mixture, and EC_{xi} is the effect concentration of the chemical i that causes the same effect as the mixture does.

The assumption behind independent action is that chemicals in a mixture do not physically, chemically or biologically interact (Bliss 1939) therefore, the effects are based on

probabilities of non-response of the organism to both chemicals in the mixture (Cedergreen et al. 2008). The mathematical expression for IA model is:

$$Y = \mu \max \prod_{i=1}^{n} qi(Ci)$$

Where Y means the biological response, Ci the concentration of chemical i in the mixture; qi(Ci) is the probability of non-response, μmax is the control response for a certain end point, is the multiplication function.

For evaluation of environmental parameters interacting with chemicals compounds, one can preview that both factors could act by independent ways. For that reason, with exceptions of some environmental variables that produces similar effects of those caused by a certain class of chemicals (e.g. oxidative stress caused by dissolved oxygen, ultraviolet radiation and metals) when the CA model can also have a applicability (Ferreira, Loureiro et al. 2008), the IA model is the most applicable in cases of natural stressor and chemical combinations assessments.

There are some mixtures that although the mode of action of its chemical components is known, show some deviations patterns from the models. These deviations are described as Synergism/antagonism when the mixture can cause a more severe (synergism) or less severe (antagonism) effect to a target organism; dose-level dependency, where the toxicity of the mixture is variable depending on whether the mixture is applied in a high or low dose, and dose-ratio deviation, which is reliant on the mixture composition, i.e. which chemical is mainly responsible for the mixture toxicity. These deviation occur when chemicals affect each others bioavailability (related to the environmental conditions), modes of action and behaviour after uptake (Cassee et al, 1998)

II. Objectives

The present work aimed to elucidate some biological behaviors and patterns regarding changes in the environment. For that, to predict interactions between natural stressors and toxicants to *Daphnia magna*, the ultraviolet radiation (UVR) and the pesticide carbendazim were chosen as the source of natural and chemical stressors, respectively and were employed in combination with each other as an example of possible stress conditions that

can be found worldwide in the environment. The first section describes the effects of ultraviolet radiation as a single stressor to lethal and sub-lethal end points of *Daphnia magna*, as survival, feeding activity, reproduction and energy budget. In the second section, it is presented the study on the combined effects of carbendazim and ultraviolet radiation on the survival, feeding activity and reproduction response of *Daphnia magna*. Third section will address the further discussions and final conclusions about results presented along the work.

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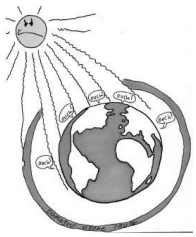

Chapter II

Effects of Ultraviolet Radiation single exposures to life parameters of *Daphnia magna*

Effects of Ultra-Violet Radiation on Survival, Feeding activity, Offspring production and Energy Budget of *Daphnia magna*

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ABSTRACT. Ozone layer is a natural Ultra-violet Radiation (UV-R) filter present in atmosphere, which has suffered serious deleterious impacts. Nowadays, although the policy for reduction on use of the ozone depleting compounds and recovery of ozone layer is showing good results, this process of healing is slow and depends on several components. The amount of UV-R that reaches earth's surface is an issue of concern among the scientific community, and has caused the execution of a number of studies to predict effects of UV-R on living organisms both aquatic and terrestrial. By taking the UV-R as a natural stressor, and considering it a threat to aquatic organisms, UV-related effects have been well reported for many zooplankton species. In this study, we used a *Daphnia magna* model organism exposed to different intensities of UV radiation (artificial source) and Photo Reactivating Radiation (PRR) simultaneously. Intensities of irradiation applied in experiments varied from 5.7kJ.m^{-2} to 31.87 kJ.m^{-2}. Immobilisation, reproduction, and feeding inhibition tests were carried out with adaptations from the already applied and described protocols. Results showed decrease on survival rates of neonates after 48h post exposure to irradiation, with a Lethal-dose LD_{50} value of 14.7kJ.m^{-2} and significant differences on feeding rates and offspring production after exposure, with Effect-Dose ED_{50} values of 14.78 kJ.m^{-2} and 21.11 kJ.m^{-2}, respectively. The energy budget of adult females after 1st brood showed a significant decrease on sugar and lipids content. The results obtained showed similar ED_{50} values, for all three different end points used (survival, feeding and reproduction), meaning that ultraviolet radiation exposure can alter the physiological status of the organism in every life-stage, with serious consequences to ecosystem functionality and food-web dynamics.

Key-words: UV-radiation, *Daphnia magna*, reproduction, feeding, energy budget

1. Introduction

Over the past decades, global concern associated to climate changes has grown as a consequence of many anthropogenic events that changed the environmental natural characteristics. One of these changes is the increase of Ultraviolet Radiation (UV-R) that reaches the earth's surface, due to stratospheric ozone depletion (Madronich et al. 1998) that can be considered a natural stressor to terrestrial and freshwater environments. Freshwater ecosystems usually have a high UV penetration, depending on their eutrophication's level, i.e., amount of dissolved organic carbon (DOC). Lakes containing low DOC absorb more UVR energy. The presence of organic particles in water can absorb the damage energy carried by short wave-lengths of UV radiation and thus, decrease the amount of energy that reach organisms, or even prevent them to be in contact with the harmful radiation (Williamson et al. 2001). It has been also observed that climate changes negatively alter the DOC concentration of adjacent lakes by altering the events of inundation and water saturation of soils and watersheds, hence increasing the transparency of lakes, that become more vulnerable to radiation input (Williamson et al. 1996). Moreover, DOC can be broken down into smaller subunits by solar UV-R, that are taken up by bacterioplankton, which can increase the transparency of lakes and thus the penetration of radiation. (Häder et al. 2007). Besides the effects of changing amounts of DOC, MacFadyen et al (2004) found that at lower temperatures, the photoprotection in ectotherms is favored rather than the temperature-dependent repair, and this factor can change the distribution of animals in the water column, with consequences to lake communities. The primary and main target of UV-R to organisms is the DNA molecule. UV-B radiation can cause dimerization of DNA bases, leading to the formation of cyclobutane pyrimidine dimmers (CPDs) and photoproducts, that block the action of DNA polymerase in repairing errors during the replication process, leading to impairments on production of basic cellular components (Buma et al. 2003).

The molecular mechanisms carried out by zooplankton to prevent UV-R damage is closely linked to UV-A radiation and visible light (approximately from 320 to 400nm) because this wavelength range provides the enzymatic repair on daphnids. The repair, defined as the PER- Photo-enzymatic Repair, is carried by the enzyme photolyase that is

activated by light and acts on the DNA repair system(MacFadyen et al. 2004). The sum of the photoprotection process and molecular repair is defined by Williamson et al (2001) as "UV tolerance".

Zooplankton also shows behavioral response to UV-R by altering their distribution through the water column as a strategy of avoidance, showing deeper distribution at daytime when higher UV-R intensity occurs. The diel vertical migration is a natural process as consequence of fish predation avoidance by zooplankton, but has been described to happen also due to increasing amounts of UV-R on the surface. These patterns can interfere with the food web dynamics of lakes (Alonso et al. 2004; Boeing et al. 2004; Fischer et al. 2006b). The physiological status of zooplankton is also changed as a consequence of combating deleterious effects caused by UV-R. Among these changes we can observe impairment on respiration rates (Fischer et al. 2006a), decrease in the number of neonates produced after irradiation exposure (Huebner et al. 2006), alteration on fecundity (Karanas et al. 1981), and decrease on feeding rates (Lacuna and Uye 2000). The metabolic costs of natural or chemical stressors to a organism is an important key for higher level response predictions, in terms of ecosystem functionality, which is expected to alter under non-favorable conditions.

Approach. In this study we aimed to evaluate the effects of UV-B radiation on life-cyle parameters of *Daphnia magna*, with the purpose to predict long-term consequences of climate changes, from organism to ecosystem level, by assessing the immediately immobilization after irradiation exposure, feeding activity, reproduction output and allocation pattern for proteins, sugar and lipids as a complement to the reproduction response.

2. Material and methods

All the experiments were conducted with the cladoceran *Daphnia magna* Straus, clone k6, originally from Belgium, and maintained in culture in our laboratory for more than 3 years. Cultures were kept in aquariums with 3L of ASTM hard water (ASTM 1980), in controlled light and temperature chambers (16h:8h light-dark), 20°C± 1°C. The green algae *Pseudokirchneriella subcapitata* was used as food source ($3x10^5$ cells/ml) together with a seaweed extract, at a concentration of 6ml/L. The cultures were renewed three

times a week. Neonates or juveniles from third to fifth brood were used in the experiments and the ones from fifth and sixth brood were used for replace old cultures. To assure test's validation, an acute test with the reference compound potassium dichromate is being performed at least twice a year in our laboratory.

2.2 UV-R experiments. To assess the UV-B radiation effects on *Daphnia magna*, all tests were performed in a controlled temperature room (20°C± 1°C), in which a UV-B apparatus was set. Ultraviolet light source was provided by an UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312nm) and two fluorescent tubes (Philips Master TL-D 18W/840) to allow photo-activating repair (PAR) during the experiments. The UV lamp was placed 30cm above the vials and clear cellulose acetate sheets (0,003mm) were used to cut-off UV-C range wavelengths. These cellulose acetate sheets were UV irradiated for 12 (twelve) hours before used in the experiments; the 12h pre-burned period has demonstrated to be suitable to minimize great differences in radiation energy throughout experiments. The exposure was made in transparent glass vials containing 500mL of ASTM hard water, placed on a high- adjustable basis below the UV lamp. Different times of exposure were used to achieve different radiation intensities (Table 1). For acute testing, UV-B intensity reaching the surface of experimental vials ranged from 5.74 $kJ.m^{-2}$ to 31.87 $kJ. m^{-2}$ while for chronic tests ranged from 5.74 $kJ. m^{-2}$ to 19.43 $kJ.m^{-2}$. Photo Reactivating Radiation (PRR), approximately 320-400nm, was held constant through all experiments. Measurements were made every hour, using an spectroradiometer placed at the surface water level and connected to a monochromator, that provide information on energy per nanometer. Espectral irradiation was obtained by the BenWin+ Software (Bentham Instruments, Reading, UK). Data transformation from $mW.m^{-2}$ to $kJ.m^{-2}$, considering the time of exposure, is shown in table 1.

2.2.3 Immobilisation tests. The acute test was adapted from the OECD guideline (OECD, 2004) using neonates <24h old exposed to different UV-B treatments. Neonates from the third to fifth brood were used in the experiments and exposed to UVR and PAR light simultaneously during 1, 2, 3, 4, 5, 6 and 7 hours, corresponding to 5.7; 10.1; 15.9; 19.4; 25.6; 30.2 and 31.8 kJ.m-2 respectively. After each irradiation time, organisms were transferred into vials with 50 ml of ASTM, and placed inside a climate chamber with a

photoperiod of 16h:8h (light-dark) and temperature of 20°C±1°C. The mean time for the transfer of the individuals from the lamp to the medium test was less than 5 minutes, and conducted under normal light conditions. For each treatment, five replicates were used, with five neonates each. After 24 and 48h (counting from the beginning of each irradiation time), immobile and dead daphnids were recorded. The end point for immobilization indicates a near future lethality and is defined as their inability to swim within 15 seconds, after gentle agitation of the test vessel (even if they can still move their antennae). Control replicates were kept in the climate chamber while the treatments received irradiation. No food was provided during both exposure and post-exposure periods.

2.2.4 Reproduction tests. Chronic tests were conducted following the OECD 211 guideline (OECD, 1998), with adaptations. For assessment of UVR effects on reproduction, neonates from the third to fifth brood were separated from the main culture and maintained in the same conditions until they complete their fourth instar, i.e. the release of the third moult, after which the beginning of egg formation take place. Daphnids within this age were exposed to the UV-lamp at the same regime described above, except for the irradiation period. The intensities applied ranged from 5.7 to 19.4 $kJ.m^{-2}$. After the exposure phase, organisms were then placed individually in 50ml glass vials, containing ASTM hard water, *P.subcapitata* as food source ($5x10^5$ cell/ml), and the algae extract, as an organic complement, and kept in a climatic chamber until they complete 21-days old. One individual per replicate and ten replicates per treatment were used. The medium test was renewed every other day; organisms were fed daily. The number of neonates was recorded and removed from the vials every day. For test validation, dissolved oxygen, temperature and pH were measured and recorded once in a week.

2.2.5. Feeding inhibition tests. Individuals with less than 24h old were separated from the laboratory cultures, and maintained at the same conditions until the release of their third moult (4-5 days old) equivalent to the fourth instars, to avoid moulting processes during the test, that are know to unstable feeding activities. The procedure for the feeding test was adapted from McWilliam and Baird (2001). Exposure to UV-R was carried without food to the ASTM medium. 4d-old *Daphnia magna* were exposed in glass vials containing 500mL

of ASTM (75 daphnias/500mL). After each time of irradiation (treatment), five individuals were transferred to 200ml vials containing 100ml of ASTM hard water and the green algae *P. subcapitata* at a concentration of $5x10^5$ cells/ml, and allowed to feed for 24h. The experimental setup included five replicates per treatment, and five individuals per replicate Controls were exposed to visible light only. For the UV-B light effects recovery, the post exposure (feeding period) was carried in a 16h:8h light-dark regime (Huebner, Young et al. 2006). To determine the initial algae concentration, a blank set of one replicate per treatment (with algae and no daphnids) was carried out. The initial algae concentration was considered as the concentration of algae in the blank vial after 24h, for each treatment. Individual feeding rates (cells/individual/h) were determined according to the method described by Allen et al. (1995)

2.2.6 Energy Budget. Following the procedure described for the reproduction test, 300 neonates were separated from the main cultures, and exposed to UV light when they reached 6-days old. The same UV intensities used for the reproduction test were applied. After each irradiation period, individuals were separated into two glass beakers of 1L contained ASTM hard water (30 individuals/beaker) and placed inside the climate chamber, until the release of their first brood. After this event, daphnids were immediately frozen in liquid nitrogen. This specific period was chosen in order to asses the effort for reproduction of *Daphnia magna* when the initial phase of recovery process from UV-B damage was being completed. For each treatment, 3 replicates of 10 organisms were used for quantifying protein/sugar, and 3 replicates for lipids quantification (Janssen 1997)

Total lipids were extracted following the methodology described by De Coen and Jansen (1997). Daphnids were homogenized in 300µL of water using a sonicator. After homogenization, 500µl chloroform (spectrofotometric grade) and 500µl methanol (spectrofotometric grade) were added and vortexed. After centrifugation, the top phase was removed and the remaining lipid extract was diluted into 500µl of sulfuric acid, and heated for 15min. at 200^{o}C. After cooled down, 1.5 µl of water was added and samples pipetted into the microplate for absorbance measurement at 375nm.

For total protein and sugar content measurements, daphnids were homogenized in 300µL of water with a sonicator. After homogenization, 15% trichloroacetic acid (TCA) was added and samples incubated at –20^{o}C for 10 min. Following centrifugation, a pellet was

formed and then washed with 5% TCA and the supernatant fractions formed were combined and used for the total sugar analysis. The remaining pellet was re-suspended in NaOH and incubated at 60^{o}C for 30 min, after which was neutralized with HCl. Absorbance was measured at 590 nm in a microplate reader; standard curves were obtained using bovine serum albumin. Total carbohydrate content of the supernatant fraction was quantified by adding 5% phenol and H_2SO_4 to the extract. After 30 min incubation at 20^{o}C, the absorbance was measured using glucose as a standard at 492nm.

2.3 Statistical Approach

The 48h Lethal Dose of 50% (LD_{50}) for UV-Radiation exposure to *Daphnia magna* was calculated using a probit analysis with the Minitab software (Minitab, 2003). For reproduction and feeding inhibition tests, data were analyzed by a one-way ANOVA, using the SigmaPlot software (Systat, 2004). Whenever data showed a normal distribution, the differences between control and treatments were obtained by the Dunnett's test. For data that failed the normality testing, a non-parametric Kruskal-wallis test was used and the multiple comparisons Dunn's method conducted. All significant differences were established at $P<0.05$. The effect-dose ED_{50} for feeding activity and reproduction was calculated using a non-linear regression, a logistic 3-parameter equation (SigmaPlot). Effect-dose ED_{10} and ED_{20} were calculated by the ToxRat professional software, (ToxRat Solutions) using a linear maximum likelihood regression and EC_x- confidence limits based on Fieller's theorem.

3. Results

Spectral composition of exposures

Spectral composition of exposures

The integrated spectrum of 1 UV lamp and two fluorescent bulbs measured below the cellulose acetate filter is given on figure 1. For data transformation (mW.m^{-2} to kJ.m^{-2}), the main value for each measurement (from 280nm to 320nm) was multiplied by 40, in order to cover the whole range of intensities during the exposure, and the value obtained

was multiplied by the time (in seconds) that lasted each exposure treatment. Data transformation and respective values are given on Table 1.

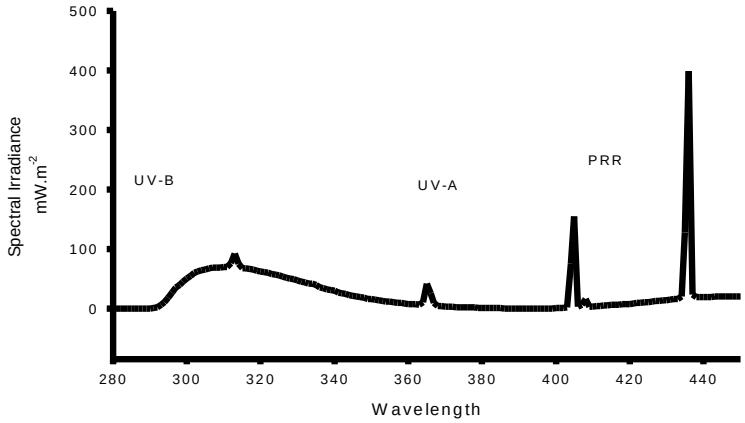

Figure. 1. Spectral composition of a UV-lamp and two fluorescent bulbs (PRR) measured at a distance of 30cm.

Table 1. Values for UV radiation intensities, presented as **kJ.m^{-2}**, applied in *Daphnia magna* exposure experiments. kJ.m^{-2} = mean value (mW.m^{-2}. m^{-2}.nm^{-1}) from 280 to 320nm x 40 **J.m^{-2}** was obtained multiplying the intensity (mW.m^{-2}.nm^{-1}) for the time of exposure in seconds.

Exposure (hours)	Survival	Feeding	Neonates*	Energy Budget^{+}
1	5.7	5.28	5.64	6.0
2	10.2	10.22	10.78	11.52
2½	-	12.54	-	-
3	14.5	14.83	15.60	17.0
3½	-	17.06	-	-
4	19.4	-	20.24	22.5
5	25.6			
6	30.2	-	-	-
7	34.8			

Acute and chronic bioassays

Immobilization records of neonates after 24h and 48h exposed to different intensities of UV-R showed decrease of survival among treatments. After 48h, no survival was observed in any of replicates of the treatments 25.6 kJ.m^{-2}, 30.2 kJ.m^{-2} and 34.8kJ.m^{-2} equivalents to 5h, 6h and 7h of exposure, respectively. The Lethal-Dose for 50% of animals calculated for this exposure was 14.78 kJm^{-2}. Control treatment showed 100% of survival.

Feeding activity, measured as feeding rate (cells/mL) also showed a decrease in values with increasing UV-intensity exposure to *Daphnia magna*. (Fig.2) Statistically differences from the control on feeding rates were detected for UV-intensity of 15 kJm^{-2}

and 17.3 kJm^{-2}, corresponding to times of exposures of 3h and 3h30min. (ANOVA, $F_{5.24}$=7.570, $p<0.05$ Dunnett's test). The ED_{50} value for feeding activity of Daphnids at 24h post-exposure was 17.88 $kJ.m^{-2}$. (st.error = 1.11; r^2=0.971). No mortality was observed during the feeding experiments neither in the controls nor in UV-treatments.

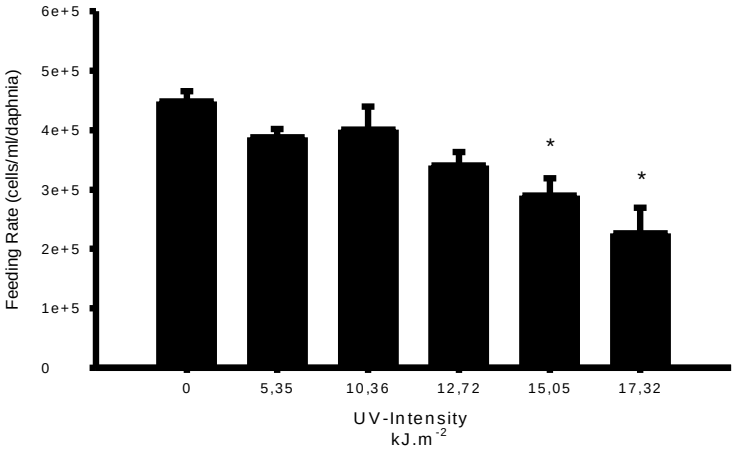

Figure 2. Effects of a pre-exposure to UV-radiation on the feeding rates of *Daphnia magna*. The feeding rates are related to the 24h post-exposure period. Data is expressed as mean values± standard errors. Asterisks indicate significant difference from the control ($p<0.05$)

The reproduction effort, measured as the number of neonates produced per live female, during 21days post exposure to UVR was detected to be different among the tested treatments. (ANOVA, $F_{4.34}$ = 10.26, $p<0.001$). Significantly differences from the control were observed from exposure of 10.1 $kJ.m^{-2}$ onwards, which corresponds to 2h, 3h and 4h of UV lamp exposure. (Fig.3) (Dunnett's method, $p<0.05$). The ED_{50} calculated for number of neonates was higher than 19.4 $kJ.m^{-2}$, which was the highest intensity used for this exposure. The length of *D.magna* at the end of the 21-days post exposure presented different mean values from the control at intensities of 14.5 $kJ.m^{-2}$ and 19.4 $kJ.m^{-2}$ (Fig.4) (ANOVA, $F_{4.34}$=7.76; $p<0.05$; Dunnett's method). UV-levels that affected the production of neonates in 10% and 20% for the organisms applied in this experiment were 6.4 $kJ.m^{-2}$ and 9.9 $kJ.m^{-2}$ respectively (ToxRat Professional)

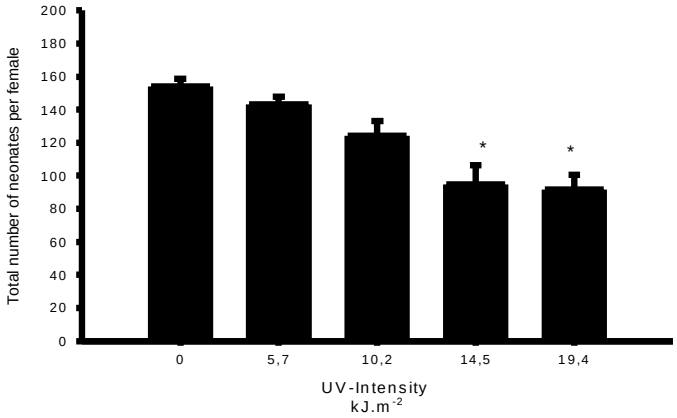

Figure 3. Number of neonates produced by *Daphnia magna* after UV radiation exposure. Data is shown as mean values± standard errors. Asterisks indicate significant differences from the control ($p<0.05$)

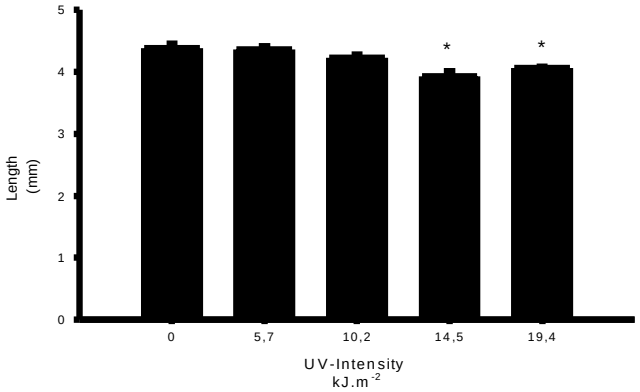

Figure 4.Body length of 21d old *Daphnia magna* pre-exposed to UV radiation. Data is shown as mean values± standard errors. Asterisks indicate significant differences from the control. ($p<0.05$)

For the analysis of the number of neonates per brood after exposure to ultraviolet radiation, a one way ANOVA was conducted for each treatment in comparison with the control, and significant differences were obtained from the second brood onwards. (Fig. 5)

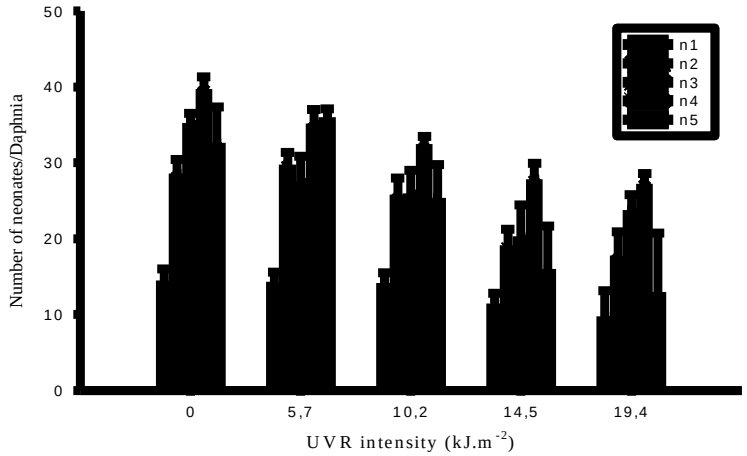

Figure 5. Mean number of neonates produced per *Daphnia magna* for each brood released after exposure to ultraviolet radiation. Data is presented as the mean value ±standard error.

Energy reserves. Sugar content, measured after the release of daphnid's 1st brood was reduced post UV-R exposure when compared to the control, being statistically significant from 10.2 kJ.m^{-2} onwards (Fig.6) (Kruskal-Wallis ANOVA, H=23.948, DF=4; p<0,001; Dunn's method p<0.05). The same pattern was observed for the lipids content (Fig.6) (Kruskal-Wallis ANOVA, H=21.01, DF=4; p<0.001 Dunn's method p<0.05)

Daphnids did not show any significant differences in their protein content post UV-R exposure after production of the first brood. (ANOVA, $F_{4.25}$=2.72, p=0.052).

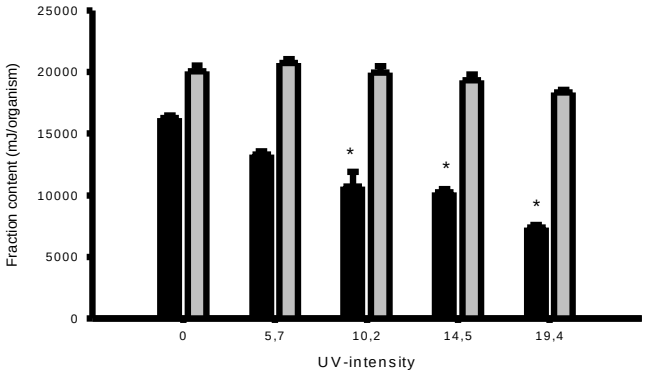

Figure6. Total sugar and protein contents of *Daphnia magna* measured after the release of first-brood upon a pre UV-R exposure. Data is shown as mean values ± standard errors, where black bars stand for sugar content and gray bars for protein contents.*represents significant differences from control. (p<0.05)

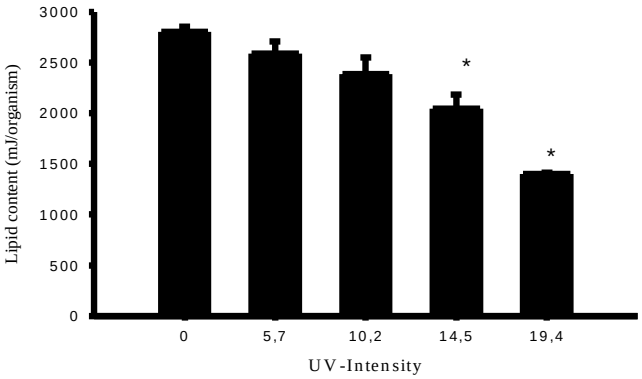

Figure 7. Total lipids in *Daphnia magna* after release of first brood upon a pre UV-R exposure. Data is shown as mean values ± standard errors. *indicates significant differences from the control ($p<0.05$)

5. DISCUSSION

To our knowledge, this is the first study to apply the OECD guidelines for immobilization and reproduction tests with *D. magna* using UV radiation as the stressor source (OECD, 2004 and OECD, 1998). Although Huebner (2006) assessed the reproductive output for the same species after irradiation exposure, the evaluation of offspring production after irradiation lasted only for twelve days. In another study survival of four species of Daphnia submitted to UV radiation and different temperatures, for a period of 9 days after irradiation, with daily feeding (Connelly et al. 2009).

In this study, we analyzed some end points that can provide knowledge on the fitness of *Daphnia magna* exposed to an artificial UV-radiation source. Our results are consistent with other studies that have demonstrated the negative impact of ultraviolet radiation on zooplankton species (Karanas, Worrest et al. 1981; Lacuna and Uye 2000; Vega and Pizarro 2000). The ED_{50} values obtained in the present study for different physiological parameters showed a similar trend, and similar values. The LD_{50} for *Daphnia magna* was lower than the ED_{50} value for feeding and reproduction. This could be explained by the age-dependent differences in sensitivity within this species. Huebner et al. 2006 found a significant decrease on the survival of *D. magna* when organisms aging from 1 to 4 days were exposed to increased irradiation. Lacuna et al (2000) also observed a stage-specific UV induced damage on the copepod *Sinocalanus tenellus* where females were more susceptible than other adults (Lacuna and Uye 2000). Moreover, the 48-h post-irradiation where no food was supplied might delay daphnids' recovery for the DNA

damage induced by UV-R exposure (Connelly, Moeller et al. 2009). Although this molecular repair is carried out by a photo-activated enzyme photolyase, and thus do not demand as much energy as nucleotide excision repair, the entire physiological status of organism plays an important role on their survival mechanisms. Zellmer (1996) found that *Daphnia pulex* presented higher survival rates to UV-radiation with higher food levels provided on the acclimation period for pre-exposure.

Considering the importance of energy status for daphnids' recovery after UV-R exposure, the feeding activity was a suitable endpoint to be applied in this study. We found lower feeding rates for *D. magna* in the 24-h post-exposure to UV-radiation, indicating a negative effect on physiological recovery process; also a momentary immobilization pattern of daphnids was noticed right after irradiation took place. Changes on swimming behavior, as a phototaxis negative effect caused by ultraviolet light was already reported for *Daphnia magna* (Storz and Paul 1998) and *Daphnia pulex* (Steams 1975) and might be an explanation for the lower feeding rates. The 24-h post irradiation period, in which organisms were allowed to feed, was carried out in the presence of PRR, which could help, beyond the feeding activity, the molecular repair of DNA. In addition, both processes (feeding effort and DNA repair) could compete for energy cost, and thus, the feeding rate is lower in high UV intensities treatment. Fisher et al. (2006) observed a stimulation of respiration rate of *Daphnia catawba* exposed to low UV-B intensity (2.08 $kJ.m^{-2}$) with simultaneous PRR, indicating a tentative of augmentation of the organism energy status. In our study, the feeding rate was significantly lower compared to the control group for the two higher doses applied, which means that the three lower doses were, at least, enough to allow a physiological ability for recovery. Data from energy budget clearly describe the effort course of the recuperation process.

In the reproduction test, it was observed that time and favorable conditions like temperature and light-cycle could improve daphnids ability to repair UV- induced damages, through molecular repair, reaching the physiological recovery status. However, significant differences were found, in number of neonates, among the three last doses in relation to the control group.

UV-R intensities corresponding to one and two hours of exposure were enough to cause reduction of neonates to 10% and 20% of the total organisms exposed. Karanas et al. (1981) also observed effects on reproduction of the copepod *Acartia clausii* that survived

after sub-lethal exposure to UV radiation, showing that even when a copepod survived exposure to UV radiation, its ability to reproduce was impaired (Karanas, Worrest et al. 1981)

Within daphnids reproduction efforts through the 21 days it was also observed that the second brood the most affected one. Eggs were produced simultaneously to the recovery process where allocation for energy resources were demanded and both metabolic process were competing for energy. The results obtained from the energy reserves content clearly exhibit the effort of energy allocation in producing offspring after stress exposure. Sugar resources were the most affected, followed by significant decrease on the lipids content. Consumption of carbohydrates is associated with faster energy demand of organism's metabolism caused by stress factors (Verslycke and Janssen 2002), because they are composed by smaller molecules that can be easily broken, releasing energy for cell metabolism. Moreover, the effects on sugar contents can suggest an intensive activity of cellular metabolism to reach stable conditions.

The results related to lipid measurements are consistent with the ones found by Tessier et al. (1983) where the increase on lipids content is linked with *Daphnia magna* survival under starvation conditions. In the present study, total lipid decreased with the increase of UV-radiation intensities, showing also, some mortality of individuals after exposure to the two highest intensities ($14.5 kJ.m^{-2}$ and $19.5 kJm^{-2}$); therefore, for these exposures, the total lipid content might also be related to survival. Another pattern of response in which lipids can be associated with is the egg and embryo production effort, as observed by Cowgill et al (1984). In our study, *Daphnia magna* was exposed to the stress source when egg formation was initiating, thus the subsequent metabolic processes for embryo development were negatively affected. Nevertheless, the higher difference in the number of neonates released was on the second brood, showing that the UV-R stress factor did not affect the production of the first brood, since the egg formation had already been initiated. On the other hand, the energy needed for the production of the second brood happened simultaneously as the recuperation process was being held.

Similar ED_{50} values obtained for survival, feeding and reproduction indicates that ultraviolet radiation can induce deleterious effects in all life stages of *Daphnia magna*, with serious ecological implications, in terms of ecosystem functionality as these effects are reported not only for zooplankton, but also for phytoplankton, (Bothwell et al. 1994;

Ekelund 1994; Cabrera et al. 1997), fishes and their larvae (Mahmoud et al.; Probst et al. 2009). We believe that applying standardized protocols to asses effects of natural stressors is an important step for analyze the effects of those stressors when interacting with chemicals compounds, by looking at the individual stress responses for later predictions of joint effects.

Ecological implications. With climate changes and decrease on the ozone layer, daphnids populations will be exposed to natural ultraviolet radiation during their life-cycle, through various life-stages as evaluated in this work. The deleterious effects described here, for each of these life-stages can conclude an impact on food-web dynamics. For instance, the depletion on feeding rates can, along time, alter algae population density, changing several physico-chemical properties of the habitat (e.g. dissolved oxygen), the total fitness status of individuals, leading to changes in daphnids populations. This event could be especially severe for shallow-water lakes, in which changes on any of the trophic components have an immediately effect on subsequent levels. (Carpenter et al. 1985; Carpenter et al. 1987; Van Donk et al. 1990).

6. CONCLUSIONS

UV intensities applied in this study have an ecological relevance, when considering the most UV affected areas in the globe. The levels of UV radiation used for exposures experiments are equivalent to UV index[1] between 1 and 2. These exposures were carried out with duration between one to five hours and results show that they jeopardize daphnids life traits like their feeding activity, mobility, reproduction and survival, in different life stages.

Considering the events of highest UV intensities in the summer and the potential increase of UV-R intensities due to climate change phenomenon and alterations on stratospheric ozone concentration, prediction effects of high UV radiation input on aquatic and terrestrial ecosystems have an important role when analyzing effects of additional environmental stressors, mainly anthropogenic pollution.

[1] The UV index is a numerical expression calculated as the mean value of effective irradiance, in $W.m^{-2}$ times 40.

Acknowledgments

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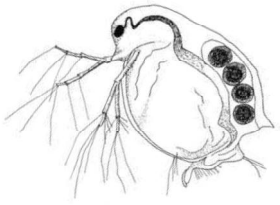

Chapter III

Toxicity predictions for combined exposures of ultraviolet radiation and carbendazim to *Daphnia magna*

Combined effects of carbendazim and ultra-violet radiation to *Daphnia magna*

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ABSTRACT. Climate change phenomenons introduce modifications on environmental components, by altering parameters such as temperature, precipitation, salinity, dissolved oxygen and ultraviolet radiation input. The later is mainly altered by depletion of stratospheric ozone layer, which increases the amount of damaging shorter wavelengths emitted by the sun, and that reach the earth's surface. In addition to ultraviolet radiation (UVR) input, the environment is dealing with the presence of chemicals originated from diverse sources, especially human activity. In the present study we investigate the effects of combined exposures of UVR and the chemical compound carbendazim to *Daphnia magna*. Immobilisation, feeding activity and reproduction tests were carried out with adaptations from already described and applied protocols. For combined experiments, daphnids received a single dose of UVR and photo-reactivating radiation (PRR) in contaminated medium, after which only chemical exposure was carried out.

For the predictions of joint toxicity, the conceptual models of independent action and concentration addition, as well as their deviations for synergism/antagonism, dose-level, and dose-ratio dependency were applied to all combination data set. EC_{50} values to carbendazim and UVR single exposures where obtained from the literature and from laboratory testing. For the combination of carbendazim and UVR, most of endpoints tested were fitted to the IA model, showing deviations to dose-ratio dependency for sub-lethal doses of UVR and carbendazim.

Keywords : *Daphnia magna,* UV-radiation, combined toxicity, carbendazim

Introduction

One of the consequences of climate changes is the potential to alter the optimum range of physical conditions of organism's surrounding environment, creating stressful situations in which their physiology processes are potentially altered. In addition, changes in physical conditions can interfere with environmental contaminants, their fate and behavior in natural systems, as well as their chemical structure or toxicology to biological systems (Noyes et al. 2009).

Chemical compounds reach the aquatic ecosystems usually by leaching of adjacent agricultural lands or by accidental discharges. They are usually characterised by their mode of action, chemistry and toxicity to non-target organisms (Jury et al. 1987; Lee et al. 2000; Hanazato 2001). The toxicity characterization of these substances takes into account effects at short and long-term exposures of organisms in controlled conditions, like temperature, photoperiod, pH and water dissolved oxygen and by these means, effects concentrations are set. (Sheehan et al. 1986; DeLorenzo et al. 2002). But, in addition to chemical exposures, real scenarios are now showing changes on their natural conditions (Hader et al. 2007) for instance, the decrease of dissolved oxygen concentration in water (Justic et al. 1996), pH alterations, increase of temperature and higher ultraviolet-B radiation (UV-B) input which are directly or indirectly caused by climate change phenomenon (Houghton et al. 1992; Parmesan and Yohe 2003; MacFadyen et al. 2004; H¨ader et al. 2007). Because of those combinations of factors occurrence in natural environments, risk evaluation of chemicals is now including more realistic situations, starting with evaluating/predicting the toxicity of more than um chemical (binary or complex mixtures) and/or with other environment variables as interaction factors that can cause different effects rather than single compounds do (Schiedek et al. 2007).

From that point of view, the ecotoxicological approach for mixtures evaluation is based on conceptual models that can describe how chemicals behave when they are present in a mixture; and that is also valid for combinations of chemical and environmental stressors. The models are based on whether compounds have the same mode of action, or different action mechanisms. Hence, they are described as Independent Action (IA), which is applied when chemicals in a mixture are believed to have different modes of action; or Concentration Addition (CA), that is used when chemicals have similar modes of action.

But there are some cases where chemical/stressors mode of action is ambiguous or unknown and both conceptual models can be used to help predict their toxicity. There are also case studies where deviations from those models occur: synergism, antagonism, or deviations for patterns that depend on the dose ratio of the mixture or the dose level applied (Jonker et al. 2005). An important characteristic of these analyses is that the prediction of all joint toxicity is always based on single compounds effects.

In the present study we investigated the lethal and sub-lethal responses of *Daphnia magna* under a combined exposure of carbendazim and ultraviolet radiation. Carbendazim (methyl-2-benzimidazole carbamate) is a fungicide belonged to the benzimidazole carbamate class, with a wide applicability in agricultural activity, against fungal diseases; it acts by interrupting the development of fungal germ tubes, the formation of appressoria and the growth of mycelia (Authority 2007). Carbendazim effects to non-target organisms have been related as impairments on cell division and inhibition of the enzyme acetylcholinesterase (Cuppen et al. 2000).

Ultraviolet Radiation is the portion of sunlight that has wavelengths beneath 440nm (280-400nm). It is usually divided in UV-A (400nm-320nm), UV-B (320nm-280nm) and UV-C (280nm-100nm). Atmospheric ozone layer is the main filter of this radiation, which prevents all the UV-C range, and part of UV-B from reaching the earth surface. Due to the degradation of the ozone layer, a significant amount of UV-B range of sunlight is reaching the earth's surface, leading to serious consequences to terrestrial and aquatic ecosystems (Houghton, Callander et al. 1992; Madronich et al. 1998; H¨ader, Kumar et al. 2007). The aquatic ecosystem is especially sensitive to the irradiation input because of its high ability of absorption. The consequences to zooplankton are related to induction of pigmentation (Rautio and Korhola 2002), changes in vertical migration, with costs to the predation dynamics of lakes (Leech and Williamson 2001; Fischer et al. 2006; Hylander et al. 2009), reproduction impairments (Karanas et al. 1981; Grad et al. 2001; Huebner et al. 2006) and oxidative stress (Borgeraas and Hessen 2000; Vega and Pizarro 2000). The main target site of ultraviolet radiation in zooplankton is the DNA molecule (Teoule 1987; Buma et al. 2003). UVR induces the formation of cyclobutane pyrimidine dimmers and pyrimidine-pyrimidone photoproducts (MacFadyen, Williamson et al. 2004). Overall, there are two types of DNA repair mechanisms: nucleotide excision repair (NER) and photo-enzymatic repair (PER) (Grossman et al. 1975). NER is a complex mechanism that requires cellular

energy from ATP, while PER is a one-enzyme mechanism, played by the enzyme photolyase which is activated by UV-A and visible light (Carell et al. 2001). Despite all physiological responses of zooplankton to this natural stress is crucial to predict environmental risk, these evaluations could also be done including anthropogenic introduced compounds such as metals and pesticides present in aquatic systems. Some previously works have studied the fate of ultraviolet radiation in organic or inorganic compounds by the photo-induced toxicity of these chemicals to aquatic organisms (Preston et al. 1999; Huovinen et al. 2001; Jung et al. 2008) So, the importance of knowing the action mechanism of the photo-inductions as well as the primordial affected compounds and effects of the combination to the organisms could be an helpful tool in employing chemicals regulation. The aim of this study was to evaluate/predict the joint toxicity of carbendazim and UVR to several and crucial life processes in *Daphnia magna*. For that, survival, feeding activity, growth and reproduction where assessed under a combined exposure of these two stressors.

Material and Methods

2.1 Test-organisms. All the experiments were conducted with the cladocera *Daphnia magna* Straus, clone k6, originally from Belgium, that have been maintained in culture in our laboratory for more than 3 years. The cultures were kept in aquariums with 3L of ASTM hard water (ASTM 1980), in controlled light and temperature chambers (16h:8h light-dark, 20°C± 1°C). The green algae *Pseudokirchneriella subcapitata* ($3x10^5$ cells/ml) and a seaweed extract (6ml/L) are added to the culture medium and the cultures were renewed three times a week. Neonates from third to fifth brood were used in experiments and the ones from fifth and sixth broods were used to replace old cultures. To assure test validation, an acute test with the reference compound, potassium dichromate, is performed at least twice a year, in our laboratory.

Single toxicity end points

Reproduction. Single exposure EC_{50} values where collected from the literature for carbendazim and ultraviolet radiation and presented in table 1. The only exception was the EC_{50} for reproduction and carbendazim exposure. For this result, a reproduction test was performed in the laboratory. Reproduction tests with *Daphnia magna* were conducted

following the OECD 211 guideline (OECD, 1998). Carbendazim used for testing had a purity of 97% (CAS No. 10605-21-7, Aldrich Chemical Corp.,USA). Neonates, from the third to fifth brood, with less than 24h-old were used for the tests. Test-medium were prepared using ASTM hard-water, and a stock-solution of carbendazim. The experimental setup included ten replicates per concentration of chemical, plus a control with ASTM and a positive control with DMSO at a concentration that did not exceed 100µg/L, algae and sea weed extract. Test medium was renewed every other day; organisms were fed daily, and the number of neonates recorded and removed from the vials every day. Physical parameters (pH, temperature and dissolved oxygen) were measured once in a week, for test validation. Less than 20% of mortality occurred in the controls. Nominal concentrations for carbendazim ranged 12.5 µg/L to 75 µg/L, respectively.

Table 1. Values obtained from single stressor exposures of ultraviolet radiation and carbendazim at different endpoints, and respectively bibliography.

End point	Ultraviolet Radiation ($Kj.m^{-2}$)	Carbendazim (µg/L)
Survival 48h LC_{50}	14.78 (0.47)[1]	156.66 (3.70)[2]
Feeding 24h EC_{50}	17.88 (1.11)[1]	97.54 (0.15)[2]
Reproduction 21d EC_{50}	>19.4	46.62 (0.90)[1*]
Reproduction 21d EC_{50}	-	50.20 (4.21)[1+]

Values for standard errors in brackets

[*]Neonates [+]Aborted eggs

[1]Data originated from previously laboratory testing (unpublished data)

[2] Data obtained from Ferreira et al. (2008)

Combination of carbendazim and UVR

Immobilisation tests. Daphnia magna juveniles with less than 24-hours were separated from the main cultures. Twenty-five neonates were separated to a glass vial (100mL volume of the test-solution) for each carbendazim concentration and controls (ASTM only). The glass vials were placed above the UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission on 312nm) and two Fluorescent tubes Philips Master TL-D 18W/840 to provide Photo Reactivating Radiation (PRR) during exposure. The irradiation time applied in these experiments was 1, 2, 3, 4, and 5 hours (irradiance values presented in table 2), after which, five neonates of each concentration and time for

UV exposure were separated in a 50mL with the respective test-chemical concentration, performing one replicate per treatment. No food was supplied. Test-vials were kept inside a controlled chamber, with the same conditions (light-cycle and temperature) of cultures. Immobilisation was recorded after 24h and 48h of exposure (that was the starting of exposure to UVR and chemical) for all chemical concentrations. Single treatments exposure of only chemical exposure and/or only UV radiation exposure were also done. Nominal concentrations ranged from 80 to 200 µg/L for Carbendazim. UV intensities ranged from 5.7 to 23.8 $kJ.m^{-2}$.

Table 2. Values for UV radiation intensities, presented as **$kJ.m^{-2}$**, applied in *Daphnia magna* exposure experiments. $kJ.m^{-2}$ = mean value ($mW.m^{-2}$. $m^{-2}.nm^{-1}$) from 280 to 320nm x 40. Values in **$J.m^{-2}$** were obtained multiplying the intensity ($mW.m^{-2}.nm^{-1}$) for the time of exposure in seconds.

Exposure (hours)	Survival	Feeding	Reproduction
1	6.34	5.28	5.64
2	12.13	10.22	10.78
2½	-	12.54	-
3	17.85	14.83	15.60
3½	-	17.06	-
4	23.54	-	20.24
5	24.52	-	-

Feeding inhibition tests. Neonates from the third to the fifth brood, with less than 24h were isolated from the laboratory cultures (3L aquarium) and placed in a 1L glass beakers (30 neonates in each beaker) and kept at the same conditions until the release of their third moult (4^{th} instars). Feeding inhibition assays were adapted from McWilliam and Baird (2001) and was composed by three phases. In the first, a combined exposure period to UV-Radiation and chemical contamination was carried out. Five glass vials containing 100mL of each concentration of test-solution, without food, with 25 daphnias each was placed at a distance of 30cm from the UV lamp. Times of exposure were 1h, 2h, 2h30min., 3h, and 3h30min, corresponding to UV intensities of 5.28, 10.22, 12.54, 14.83 and 17.06 $kJ.m^{-2}$ respectively. In a second phase, the feeding period and after the irradiation period, five

daphnids from each concentration were placed in a 200ml vial containing 100mL of the test-solution (same concentration of exposure) and the green algae *Pseudokirchneriella subcapitata* ($5x10^5$ cells/mL). The feeding period lasted for 24-hours and was carried out on light conditions, in order to provide the conditions of photo-repair radiation and avoid mortality of daphnids. A blank control of algae (no daphnids) was performed and used as the initial algae concentration after 24h. The third phase was the post-exposure period where daphnids were transferred to a "clean" medium (ASTM + algae) in 50mL glass vials, and allowed to feed for four hours. Following feeding and post-exposure period, absorbance measurements of the medium were taken. Single stressors exposures for carbendazim and UVR were also carried out in simultaneously. One replicate was performed per combination treatment, and two replicates for the single treatments. The solvent control was not used in the combined experiment approach with UV radiation, because previously results have show no significant differences between ASTM and ASTM with 100µ/L of the solvent, and also mainly due to small space facilities under the UV-Lamp. Nominal concentrations of carbendazim used on combinations varied from 125 µg/L to 225 µg/L.

Reproduction Test. The chronic test using *Daphnia magna* was adapted from OECD 211 protocol (OECD, 1998). Neonates (<24h old) were placed in 50mL glass vials (one neonate/vial) in each and food (*P.subcapitata* and seaweed extract). At 6-days old (when daphnids started to show the egg mass) they were exposed to UV-Radiation, in 100ml glass vials, containing the corresponding test solution treatment. Each treatment had four individuals, which correspond to one daphnia/time of exposure/concentration. Also, a UV-control treatment was performed, including four daphnids in only ASTM exposed to the lamp (one replicate/time of exposure). The exposures to UVR were complete at the same time, and in the post-radiation regime, daphnids were placed in the same conditions pre-exposure. In the day followed exposure, test-medium was renewed and occasional mortality and any abnormalities of daphnids recorded. Onwards, test medium was renewed every other day. Organisms were fed daily with the green algae *P.subcapitata* ($3x10^5$cells/mL) and seaweed extract and the number of neonates and/or aborted eggs was recorded every day. Nominal concentrations of carbendazim ranged from 12.5µg/L to 75µg/L. At the end of the test, the length of daphnids was measured using a stereomicroscope.

Data analysis.

In order to detect significant differences on the number of neonates produced at the end of 21 days, reproduction data were analyzed by a one-way ANOVA with the SigmaStat Software, followed by the Dunnett's or Dunn's method to detect differences towards control. (Systat, 2004). When data did not present a normal distribution, a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed, and the Dunn's test conducted to detect the differences from the control treatment. For carbendazim, a solvent-control treatment was used, and differences between positive and negative control were assessed by a t-test or a Mann-Whitney Rank test when data didn't show a normal distribution. The Effect-concentration that reduced in 50% the number of neonates produced (EC_{50}) was calculated by a Non-Linear Regression, with a Logistic 3-parameter equation, using SigmaStat Software. The Lowest Observed Effect concentration (LOEC) and No-Observed Effect concentration (NOEC) for both chemicals tested were also calculated by multiply comparisons test (Dunn's or Dunnett's test). The significances were established at p 0,05. In order to validate combination assays, the EC_{50} of single controls (single chemical and single UV-exposure) used in combined toxicity tests were calculated by the same procedure described above.

To calculate/predict the joint toxicity of carbendazim and UVR exposures, the response profile for UVR was first described, and the information was used to calculate a full factorial experimental design for combinations. The expected effect of combinations was based on the dose-response curve for each of the components and the results of the combinations. For that, the MixTox model described by Jonker et al. (2005) was used, by applying the reference model Independent Action (IA), as well as the possible deviations from the model "S/A, "DL" and "DR" dependence, which were obtained by an addition of two parameters (*a* and *b*) (Table 3). For analysis of carbendazim and ultraviolet radiation, based on their modes of action, only the IA model was used to describe the data for acute, feeding and reproduction endpoints. After fitting our data, the best adjustment was analysed using the method of maximum likelihood, and the best fit was chosen to describe the data (Jonker, Svendsen et al. 2005)

Table 3. Interpretation of additional parameters substituted into the independent action model (IA) reference model that define the functional form of the deviation patter

Parameter	Value IA	Meaning
	Synergism/Antagonism	
a	>0	Antagonism
	<0	Synergism
	Dose ratio dependence	
a	*>0*	Antagonism, except for those mixtures ratios where significant negative *b indicate synergism*
	<0	Synergism, except for those mixture ratios where significant positive *b* indicate antagonism
b_i	>0	Antagonism where the toxicity of the mixture is caused mainly by the toxicant *i*
	<0	Synergism where the toxicity of the mixture is caused mainly by toxicant *i*
	Dose level dependence	
a		
	>0	Antagonism low dose level and synergism high dose level
	<0	Synergism low dose level and antagonism high dose level
b_{DL}	>2	Change at lower dose level than the EC50
	=2	Change at the EC50 level
	$1< b_{DL} <2$	Change at higher dose level than the EC50
	<1	No change, but the magnitude of synergism/antagonism is dose level (CA) or effect level (IA) dependent
EC50 = median effect concentration		

*Adpated from Jonker et al (2005)

Results

Chemical Analysis

To assess contamination accuracy, CBZ analyses were made and the results showed that measured concentrations varied generally between 38% and 2%, depending on the dose analysed. All calculations were based on nominal concentrations.

Chronic Single toxicity tests

There was no significant difference between the positive and negative controls (p=0.15); therefore, we used the negative control in the comparisons with other treatments. The concentration of carbendazim that causes 50% of reduction on offspring production of *Daphnia magna* was 46.62µg/L (SE=0.90) (Fig.1). LOEC and NOEC values for carbendazim were 50µg/L and 37.5µg/L, respectively. Also, at the two highest concentrations, daphnids did not produce any neonates. Table 1 shows the values of EC_{50} 48h and 24h for survival and feeding activity of *Daphnia magna* exposed to carbendazim, obtained from Ferreira et al. (2008). An important figure observed and reported for carbendazim exposure were the significant production of aborted eggs in the three highest concentrations (fig.2). The EC_{50} value for the number of aborted eggs was 50.20 µg/L (SE=4.21). Significant differences from the control on mean numbers of neonates produced and aborted eggs were found at concentrations of 50 µg/L, 62.5 µg/L and 75 µg/L ($p<0.05$; Dunn's Method). The mean-size of daphnids at the end of 21-days showed significant differences from the control group at 62.5µg/L and 75 µg/L (Fig.3) ($p<0.05$; Dunnett's method). Test-medium parameters were measured at the beginning, middle and end of the test and varied from 8.0mg/L to 8.8mg/L for dissolved oxygen, 19°C to 21°C for temperature, and pH was around 8.0 (measurements taken from old medium).

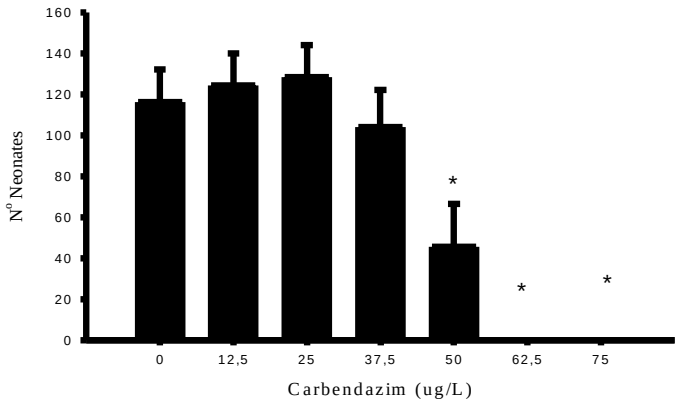

Figure 1. Number of neonates cumulative produced by *Daphnia magna* exposed to carbendazim for 21 days. Data is shown as mean values ±standard errors. Asterisks indicates significant differences from control ($p<0.05$)

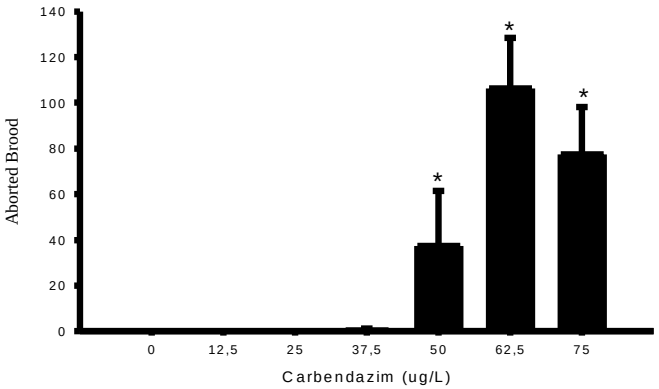

Figure 2. Number of aborted eggs produced by *Daphnia magna* during exposure to carbendazim after 21 days. Data is shown as mean values ±standard errors. Asterisks indicates significant differences from control ($p<0.05$)

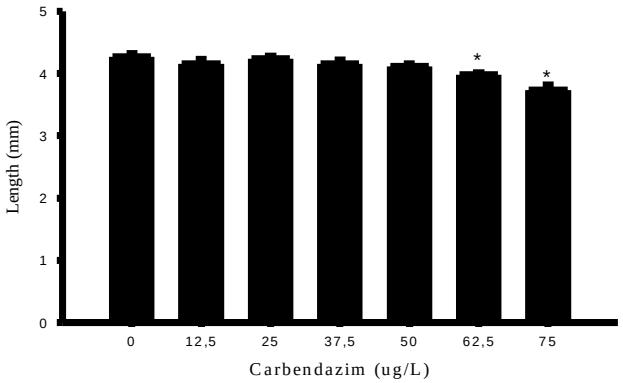

Figure 3. Body length of 21 d old *Daphnia magna* exposed to carbendazim. Data is shown as mean values± standard errors. Asterisks indicate significant differences from the control ($p<0.05$).

Binary combinations of carbendazim and UV-Radiation

To obtain a toxicity response for combination of chemical and natural stressors, the conceptual model for independent action (IA) was applied based on the single dose-response pattern of both stress factors. LC_{50} and EC_{50} values from literature and the one found in this study were crucial to build the experimental setup for combined exposures (table 1).

Data originated from combination of carbendazim and UV radiation for different end points were analysed by using the independent action model, based on the mode of

action of carbendazim and UVR as single stressors. Survival data fitted significantly to the IA model (SS=13.57, r^2=0.91, p<0.05). Adding parameter *a* to check whether there was a deviation for synergism or antagonism, the SS value decreased (SS=12.38) but not significantly ($p(X^2)$ = 0.27), indicating no evidences for significant deviations for synergism or antagonism. Adding parameter *b*, to test for dose-level or dose ratio deviations, none of the p values were significant enough to describe a deviation. The best fit for the survival data set was the independent action. (Fig. 4*a* and 4*b*).

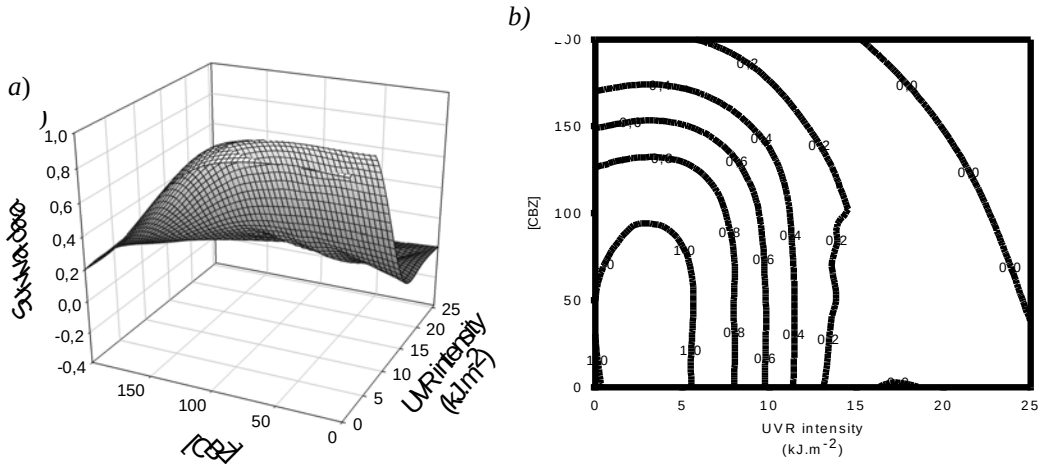

Fig. 4. Dose-response relationship for the combination of carbendazim and ultraviolet radiation to the survival of Daphnia magna, showing an adjustment to the IA model; *a* represents the 3-D mesh, and *b* the isobols surfaces.

For feeding inhibition exposure, the IA model fitted also our data (SS= $9.7x10^{-10}$, r^2=0.85, p=$1.96x10^{-15}$). Adding parameter *a* to the equation, the r^2 value was increased (r^2=0.88), and the SS value decreased, with significant adjustment to antagonism. (SS= $7.81x10^{-10}$, *a*= 1.90 p=0.002). The dose-ratio deviation from S/A was also tested by adding parameter *b* and was the best description for the data (Fig. 5*a* and 5*b* (r^2=0.93, SS=$3.34x10^{-10}$, p=$3.04x10^{-8}$). The toxicity of this combination was mainly caused by the UV radiation, as indicated by the interpretation of additional parameters *a* and *b* (*a*=-10.35, *b*=23.69). The EC_{50} of carbendazim for feeding rate was decreased at one and two hours of exposure to UVR (6.5kJ.m^{-2} and 12.8 kJ.m^{-2}) (table 4).

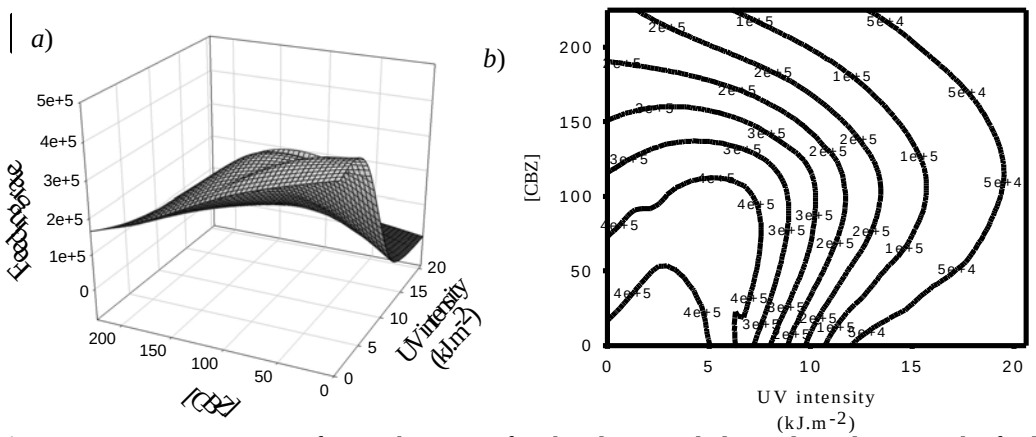

Fig. 5. Dose-response pattern for combination of carbendazim and ultraviolet radiation to the feeding activity of *Daphnia magna*, showing a dose-ratio deviation from the IA model. a represents the 3-D mesh, and b the isobols surfaces.

In relation to the reproduction output response for this exposure, there was a good adjustment of data set to the IA model (SS= 2929.2, r^2=0.95, p=1.29x10^{-13}). The addition of parameter *a* did not show a significant deviation for synergism/antagonism from the IA model, even though reduced the SS value (SS=2783.10, p=0.25). However, adding parameters *a* and b_{DR} decreased the SS significantly (SS=2005.49, r^2=0.96, p=0.007). Therefore, a dose-ratio dependent deviation from independent action was concluded (*a*= 27.58 and *b*=-450.8) (Fig. 6*a* and 6*b*). In this case, synergism occurred when the toxicity of the combination is mainly caused by UV radiation.

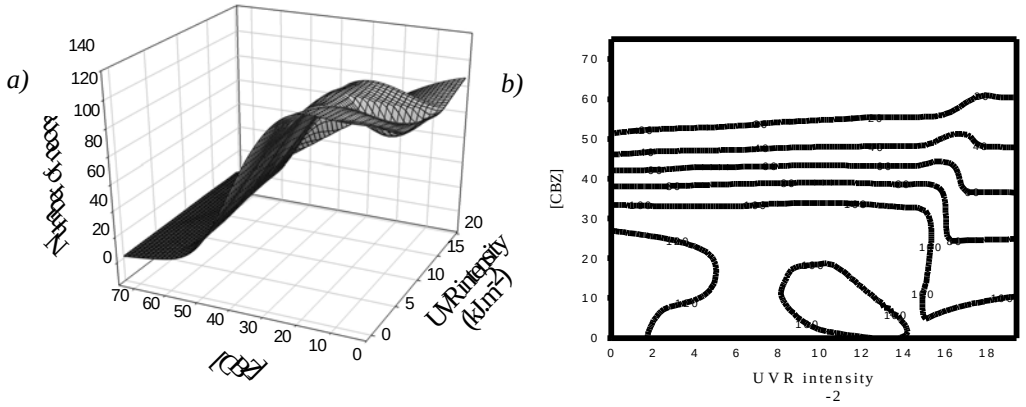

Fig. 6. Dose-response pattern for combination of carbendazim and ultraviolet radiation to the reproduction output of *Daphnia magna*, showing a dose-ratio deviation from the IA model. a represents the 3-D mesh, and b the isobols surfaces.

Discussion

Single exposures

Chronic exposure of *D.magna* to carbendazim in this study showed an EC_{50} value of 46.62µg/L and Van den Brink et al. (2000) found a similar value of 37 µg/L for 28-days of exposure. Carbendazim is known as a disruptor of the mitosis process in plants and spermatogenesis in mammalian cells. The mode of action is mainly the interference of formation or functioning of cell's microtubules (Davidse 1977). In this study, besides the reduction on number of neonates with increasing concentrations of carbendazim, a large number of aborted broods were observed at the three highest concentrations. This fact could be due to impairments of egg development, by disruption of cell division. Kast-Hutcheson et al. (2001) have demonstrated that the fungicide propiconazole negatively affects the embryonic development of *D.magna*, at concentrations ranging from 0.06 mg/L to 0.12mg/L showing the occurrence of aborted eggs during different development stages. In the study of Ferreira et al. (2008) carbendazim was shown to reduce in 50% the feeding activity of *D.magna* at a concentration of 100µg/L (EC_{50} value present in table 1). Slijkerman et al (2004) also found impairments on feeding activity of *D.magna* exposed to carbendazim in *in situ* experiments, at levels of 300µg/L of the chemical. Based on these observations, we can not exclude the hypothesis that reduction on number of neonates observed in this study can be was also related to decrease in food uptake. In previously experiments with single UV exposures conducted in our laboratory using *Daphnia magna*, we found that ultraviolet radiation reduce in 50% the feeding activity of daphnids at a dose of ~18kJ.m^{-2}, which corresponds to ± 3 hours of exposure to the UV lamp. The reproduction output was also impaired by UVR, with a dose-effect of ~20 kJ.m^{-2}, corresponding to ± 4 hours of exposure (unpublished data). Furthermore, 48h-ED_{50} values for ultraviolet radiation and LC_{50} value for carbendazim to *D. magna* were established at 14.7 kJ.m^{-2} (unpublished data) and 156.66 µg/L (Ferreira et al. 2008), respectively.

Combined exposures

Recently, several studies are addressing the toxicity of chemicals in interaction with natural stressors (Preston, T.W.Snell et al. 1999; Huovinen, Soimasuo et al. 2001;

Schiedek, Sundelin et al. 2007; Wang et al. 2008). The main concern is whether natural variables increase chemical toxicity present in the environment.

In acute experiments with carbendazim and ultraviolet radiation, data fitting to the IA model was based on the assumption of independently probabilities of response to both stressors. No deviations from the model meant that the carbendazim and UVR had no relations and/or competition for target sites in the organism at lethal doses; however, their single effects independently from each other were combined to cause negative physiological effects on the organisms, traduced in an additive toxicity.

Nikkila et al. (1999) observed that the acute toxicity of pyrene to *Daphnia magna* was increased under environment UV-B levels, showing a positive correlation between the EC_{50} of pyrene and the amount of DOC presented in the test-water. When exposed *Daphnia magna* to sulfathiazole and different UV-B intensity levels, Kim et al (2009) found that the generation of reactive oxygen species and consequently oxidative stress in the organism was higher in the combined exposures than in the single treatments with only sulfathiazole, as well as increased mortality rate of daphnids under combined stressors exposure, suggesting a higher toxicity of this chemical when ultraviolet radiation was present.

Table 4. 24-h and 4-h post-exposure feeding inhibition EC_{50} obtained for carbendazim at different UVR intensities

UVR intensity	EC_{50} *	EC_{50} +
0	179.87	115.86 (6.69)
6.5	79.53 (84.0)	165.38 (2.44)
12.8	164.33 (27.88)	85.35 (230.6)

*24-h exposure +4-h post-exposure

Table 5. 48-h LC_{50} obtained from nominal concentrations of carbendazim at different UVR intensities

UVR intensities ($kJ.m^{-2}$)	0	5.7	10.2	14.5
carbendazim	151.07 (11.05)	176.91 (13.62)	77.44 (45.47)	<

Standard errors in brackets.

For feeding activity response, a dose-ratio deviation for antagonism was observed, where the toxicity of the combination is mainly caused by the UV radiation. At higher UVR exposures, the feeding rate was zero. For this combination, as predicted by the interpretation of additional parameters *a* and *b*, the decreased in feeding activity of *Daphnia magna* was mainly caused by the ultraviolet radiation. Ferreira et al (2008) obtained an antagonistic response for the combination of low dissolved oxygen (DO) and carbendazim to the feeding activity of *D.magna*; however, it was not accepted because the calculation of EC_{50} values of carbendazim at different DO showed synergistic effect. Ultraviolet radiation has been shown to interfere with feeding of zooplankton (Lacuna and Uye 2000). In this experiment, we observed a decrease in movement of the appendage of the organisms after irradiance period, which could explain the impairment on daphnid's feeding activity, which is also supported by Lampert (1987) that observed that feeding activity in *Daphnia* is dependent on the thoracic appendages movements.

For reproduction, a dose-ratio deviation was observed for carbendazim and UVR combination, suggesting less toxicity of carbendazim than expected, except when the ultraviolet radiation is the dominant item in the combination i.e., at low concentrations of carbendazim and high ultraviolet radiation; EC_{50} values calculated for carbendazim at different radiation intensities are presented in table 6 and indicates this pattern. As mentioned before, the mechanism in which carbendazim alters the reproduction response of *D.magna* is by means of impairment on cellular division; while for ultraviolet radiation there are related effects on reproduction (Huebner, Young et al. 2006; Gillespie 2008), the specific mechanism in which UVR alters the offspring production by *D.magna* is not described until now. For that reason, we can not establish any integrated effects for the combination of these two stressors, besides independent probability of response by the organism to both components.

Table 6. 21-days EC_{50} Values obtained from nominal concentrations of carbendazim at different UVR exposures

UVR intensity	EC_{50} *	EC_{50} +
0	40.05 (1.36)	49.19 (1.31)
5.7	44.63 (1.92)	42.73 (1.86)
10.2	47.19 (3.56)	54.0 (3.28)
14.5	44.77 (5.03)	44.44 (1.74)

Values for standard errors in brackets. *Neonates, + Aborted brood

All the described patterns of response for the combination of UVR and carbendazim in this study were not related to the photo-modification of the chemicals (photo-induction or photo-degradation) because of the implications established by the experimental setup applied. As daphnids were exposed to chemical and ultraviolet radiation for a period of maximum four hours, and the rest of the experiments were conducted only in chemical exposure, we believe that even if degradation or a toxicity induction of the chemicals was happening, this was not sufficient to induce effects on daphnids. However, the dose-response patterns of the combinations were observed at the organism level, in which the toxicokinetic phase (process of uptake, distribution and excretion of chemical) and/or toxicodynamics (effects of stressor in the receptor, cellular target or organ) might be somehow altered by interaction between different stress factors.

Acknowledgments

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Chapter IV

General discussion and conclusions

Final remarks and conclusions

The main established purpose of this study was to address single effects of ultraviolet radiation and carbendazim on *Daphnia magna*, and use these results as a support tool to predict effects of combined exposures to carbendazim and ultraviolet radiation simultaneously.

First highlights that could be addressed from the second chapter results are the similarities among the effect-dose values of ultraviolet radiation for different endpoints tested. As previously discussed, this fact is due to the age-dependent sensibility of *D.magna* to ultraviolet radiation, demonstrated by Huebner (2006) when exposed daphnids from 1-4d old, to radiation intensities varied from 3.4 $kJ.m^{-2}$ to 6.8 $kJ.m^{-2}$, which are within the radiation range used for our assays. Lacuna and Uye (2000) found the same pattern for the copepod *Sinocalanus tenellus* exposed to ultraviolet radiation, and related this stage-specific sensibility to differences in integumental photoprotection, pigmentation and the content of UV-absorbing compounds present in the carapace. An additional outstanding observation was the importance of visible and UV-A light, referred as photo reactivating radiation (PRR), to the recovery process of organisms pre-exposed to irradiation. This range-light provides energy for the action of photolyase, which repairs the damage caused to DNA molecule. (Carell, Burgdorf et al. 2001). In natural environments, daphnids tend to avoid ultraviolet radiation by migrating into deeper waters (Fischer, Nicolai et al. 2006b). It is important to asses whether this migration pattern interferes with the amount of UV-A and visible light received by the organism, once the exposure to damaging irradiation (shortest wavelength) had occurred.

Dissolved organic carbon (DOC) has been reported as playing an important role in lake's transparency to ultraviolet radiation input, since the partitioned fraction of organic matter absorbs most of damaging energy of UV rays, preventing deeper layer water and its living organisms from receiving the irradiation (Williamson, Neale et al. 2001). Indeed, lakes with high DOC concentrations might experience decreases in dissolved oxygen concentration due to high metabolic rates of microorganisms (Anusha and Asaeda 2008), which in turn, represents also a natural stressor to zooplankton species (Nebeker et al. 1992). For that reason, besides combinations of chemicals and natural stressor, the need of combinations of both natural stressors, dissolved oxygen and ultraviolet radiation, for

instance, is possible to provide information on the degree of concern from which environmental risk evaluation should start.

The third Second chapter addresses the combined exposures of toxicants and UVR. The first important point to be considered from these results is that observed effects post-ultraviolet exposures were not related to any photo-toxicity phenomenon, because of the manner in which the experimental model was set up. Even if chemical degradation had occurred, it was not reflected in the toxicity of the compound to the organism, because contaminated medium was renewed every other day, without further UVR exposure. For *Daphnia* species, the reciprocity phenomenon related to UVR is not hold (Grad, Williamson et al. 2001) which means that effects of different dose rates on survival and reproduction might not yield similar trends, even when the total UV dose is the same. This is explained by the presence of photoenzimatic repair (PER) of the DNA molecule, that keeps up with the damage caused by the irradiation. For that reason, data presented in the third chapter were analysed from the single-dose input, for the ultraviolet radiation, and continuous exposure to chemicals. It might happen that in natural environments, the more realistic situation would occur through the other way around: small doses of UV during larger periods of time. Therefore, from these experiments, we assessed the response-pattern for combinations of a single high dose of UV-B and constant chemical exposure; a situation that can be easily experience by populations living at high altitudes and clear water systems.

Our results from these combinations, after analysed by the reference models, were consistent to follow the independent action assumption, with two deviations cases to dose-ratio dependency, suggesting less toxicity for chemical when the ultraviolet radiation was dominant, which was associated induced effects of UVR; indeed, no pattern of interaction between chemicals and radiation were established.

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